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A Novel Phosphodiesterase Type 4 Inhibitor, YM976 (4-(3-Chlorophenyl)-1,7-diethylpyrido[2,3-*d*]pyrimidin-2(1*H*)-one), with Little Emetogenic Activity

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ABSTRACT

We synthesized a novel phosphodiesterase type 4 (PDE4) inhibitor, YM976, that is structurally different from the other PDE4 inhibitors like rolipram. In the present study, the pharmacological profile of YM976 was investigated. YM976 exhibited a strong and competitive inhibition against PDE4 purified from human peripheral leukocytes with an IC_{50} of 2.2 nM. IC_{50} values of rolipram and RP73401 were 820 and 0.43 nM, respectively. Test compounds had no effects on the other PDE isozymes, PDE1, -2, -3, and -5. YM976 potentiated prostaglandin E_2-induced cAMP accumulation in a human mononuclear cell line, U937, and inhibited tumor necrosis factor-α production from human peripheral blood mononuclear cells stimulated by lipopolysaccharide. Anti-inflammatory activities of PDE4 inhibitors were compared in rat carrageenan-induced pleurisy models. YM976, rolipram, and RP73401 inhibited the cell infiltration into the pleural cavity with oral ED_{30} values of 9.1, 10, and 7.4 mg/kg, respectively. YM976 produced no emesis up to 10 mg/kg, whereas rolipram and RP73401 induced emesis at oral doses of 3 mg/kg. To evidence the dissociation of anti-inflammatory activity from emesis, the anti-inflammatory effect of YM976 was examined in ferrets. YM976 dose dependently reduced carrageenan-induced leukocyte infiltration at the doses of 1, 3, and 10 mg/kg, p.o. On the other hand, rolipram failed to show obvious inhibition at doses that do not induce emesis. In conclusion, YM976 is a novel and orally active PDE4 inhibitor and possesses a good separation of emetogenicity from anti-inflammatory activity.

The cyclic nucleotide phosphodiesterases (PDEs) are a family of enzymes that play an important role in regulating intracellular levels of cAMP and cGMP by catalyzing the hydrolysis of cyclic 3′,5′-adenosine and guanine nucleotides to the corresponding nucleotide 5′-monophosphates. PDEs are classified into at least seven isozymes on the basis of their functional characteristics such as substrate specificity and responsibility to selective PDE inhibitors (Banner and Page, 1996). Among the enzymes, type 4 PDE (PDE4) is a high-affinity cAMP-selective isozyme, and is predominantly contained in immune and inflammatory cells (Teixeira et al., 1997). Inhibition of PDE4 activity in vitro results in the elevation of intracellular cAMP level, which in turn leads to functional inhibition of eosinophil (Souness et al., 1995), macrophages (Goncalves de Moraes, 1998), neutrophils (Anderson et al., 1998), mast cells (Shichijo et al., 1999), basophils (Shichijo et al., 1997), monocytes (Cohan et al., 1996), and lymphocytes (Essayan et al., 1997).

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Recently, several selective PDE4 inhibitors, including rolipram, CDP840 (Holbrook et al., 1996), CP-80633 (Wright et al., 1997), and RP73401 (Souness et al., 1995) have been developed, and clinical trials are already showing the potential use of these PDE4 inhibitors in asthma and chronic obstructive pulmonary disease (COPD) (Torphy et al., 1999). Unfortunately, the anti-inflammatory effect of PDE4 inhibitors has been considered to be associated to some extent with nausea and vomiting as adverse effects (Banner and Page, 1995). Clinical usefulness of PDE4 inhibitors is limited mainly due to this adverse effect. PDE4 inhibitors with little or no emetogenicity have been desired for a novel anti-inflammatory agent. Although some PDE4 inhibitors were reported to be less emetic, no compounds have been evidenced to show dissociation of emesis and anti-inflammatory activity in vivo.

We have found a novel PDE4 inhibitor, YM976 (see Fig. 1), which was synthesized based on a lead compound found by random screening. YM976 is a pyrimidine derivative and

ABBREVIATIONS: PDE4, phosphodiesterase type 4; YM976, 4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-*d*]pyrimidin-2(1*H*)-one; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; PBMC, peripheral blood mononuclear cells; CIP, carrageenan-induced pleurisy; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; MC, methylcellulose.

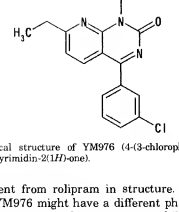

Fig. 1. Chemical structure of YM976 (4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-*d*]pyrimidin-2(1*H*)-one).

totally different from rolipram in structure. Thus we expected that YM976 might have a different pharmacological profile from rolipram and the existing inhibitors. In the present study, we elucidate the pharmacological profile of YM976 using several in vitro and in vivo models and illustrate the apparent dissociation of anti-inflammatory effects from emetic effect in the same animal species.

Materials and Methods

Animals. Male rats weighing 130 to 135 g were purchased from SLC (Hamamatsu, Japan), and male ferrets weighing 0.65 to 1.15 kg were purchased from Charles River, Japan. All the animals were maintained in ordinary animal cages under a constant 12-h light/dark cycle. Rats and ferrets were housed in groups of six and one per cage, respectively. Food and water were available ad libitum.

Chemicals. YM976 (4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-*d*]pyrimidin-2(1*H*)-one), rolipram, RP73401, CDP840, SB207499, and cilostamide were synthesized by the department of Chemistry, Yamanouchi Pharmaceutical Co., Ltd. (Tsukuba, Japan). In experiments in vitro, all drugs were dissolved with dimethyl sulfoxide (DMSO), and the final concentration of DMSO was less than 0.1%. In experiments in vivo, all drugs were suspended with 0.5% methylcellulose (MC) solution, and were orally administered in a volume of 3 ml/kg. Control groups were treated with each corresponding vehicle.

The reagents and chemicals used were DMSO, methanol, diethylether, and chloroform, purchased from Kanto Chemical Co. (Tokyo, Japan) and Bis-Tris, EGTA, cGMP, cAMP, carrageenan, formylmethionyl-leucyl-phenylalanine, HEPES, sodium acetate, Tris-HCl, $MgCl_2$, 2-mercaptoethanol, phenylmethylsulfonyl fluoride, pepstatin A, leupepin, aprotinin, and benzamidine from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Rockville, MD), MC (TC-5E) from Shin-Etsu Chemical Co. (Tokyo, Japan), and dextran and Ficoll solution from Amersham Pharmacia Biotech (Uppsala, Sweden).

Isolation of Human Peripheral Leukocytes. Leukocytes were isolated from peripheral blood of healthy human volunteers. Physiological saline supplemented with 3% dextran was added to heparinized human peripheral blood, and the mixture was incubated for 40 min at 37°C to precipitate erythrocytes. The supernatant after precipitation of erythrocytes was recovered and washed once with phosphate-buffered saline, and the pellet, which contained leukocytes, was resuspended in a buffer (pH 7.4) containing NaCl (140 mM), KCl (5 mM), glucose (5 mM), and HEPES (10 mM).

Isolation of Human Peripheral Blood Mononuclear Cells (PBMC). The suspended leukocytes were overlaid on a solution for density gradient centrifugation use (Ficoll solution) and then centrifuged at room temperature for 40 min at 450*g*, thereby separating mononuclear cells and granulocytes. Mononuclear cell fraction was washed once and resuspended in RPMI 1640 medium with 10% FBS.

Purification of PDE Isozyme. PDE2, PDE3, PDE4, and PDE5 were prepared from human peripheral leukocytes, and PDE1 was prepared from rat ventricles. All enzymes were partially purified by Q Sepharose Fast Flow (Pharmacia Biotech, Sweden) with 0.05 to 1.25 M sodium acetate gradients from 100,000*g* supernatants. The following protease inhibitors were maintained throughout: phenylmethylsulfonyl fluoride (50 μM), pepstatin A (5 μM), leupeptin (40 μM), aprotinin (20 μM), and benzamidine (2 mM). Characterization of each PDE isozyme was recognized as follows: PDE1, Ca^{2+}/calmodulin-activated; PDE2, cGMP-activated; PDE3, cGMP-inhibited; PDE4, cAMP-specific and inhibited by rolipram; PDE5, cGMP-specific.

Cloning of PDE4$_A$. To obtain the full length of PDE4A, reverse-transcription-polymerase chain reaction (RT-PCR) was performed. RT-PCR was carried out using specific primers and the human brain cDNA as a template for 35 cycles at 96°C for 30 s, 60°C for 30 s, and 74°C for 3 min. Specific primers were synthesized based on the report (Bolger et al., 1993). Forward primer (5′-TGTAGGTTG-GAAGGGC-3′) corresponds to nucleotides 66 to 81 and reverse primer (5′-TTGAGGCAGAGGAGGAGGTG-3′) to nucleotides 2837 to 2856. For RT-PCR, human brain cDNA was converted from human brain poly(A)$^+$ RNA (Clontech, Palo Alto, CA), using random hexamer primer with the first strand cDNA synthesis kit (Clontech). PCR product was subcloned into a plasmid vector, pCRII (Invitrogen, San Diego, CA), and sequenced. After sequence confirmation in several clones, the insert of clone 8 was subcloned into pEF-BOS expression vector. This vector was kindly provided by Dr. Shigekazu Nagata (Osaka Bioscience Institute, Japan) (Mizushima and Nagata, 1990).

Determination of the Mode of Inhibitory Action Using PDE4$_A$. To clarify the mode of inhibitory action of YM976, we achieved the PDE inhibitory assay using recombinant PDE4A. In this assay, we used 0.5, 1, 2, and 4 μM cAMP as the substrates and examined the effects of YM976 at the concentrations of 0, 1.5, 3, 6, and 12 nM. The mode of inhibitory action of YM976 was assessed by plotting according to Lineweaver-Burk and Dixon.

Determination of Inhibitory Effect of Compounds for PDE. A predetermined amount and concentration of each test compound was incubated at 30°C for 10 min in a reaction mixture (pH 8.0) containing Tris-HCl (40 mM), $MgCl_2$ (5 mM), 2-mercaptoethanol (4 mM), cAMP (1 μM), [^{3}H]cAMP (10 nM), and a PDE stock solution. The mixture was placed in boiled water for 1 min, cooled in an ice bath, mixed with 1 unit of 5′-nucleotidase, and then incubated at 30°C for 10 min. The reaction was stopped by the addition of 1 ml of methanol. The solution was passed through a Dowex 1-X8 (Bio-Rad, Richmond, CA) column to adsorb unhydrolyzed material, and then the radioactivity in the elution was measured.

Measurement of Intracellular cAMP Elevation in U937 Cells. cAMP elevation studies were conducted in human monocytic cell line U937. Cells (0.25×10^6/250 μl) resuspended in Krebs' buffered solution were incubated at 37°C for 15 min in the presence of test compounds or vehicle (250 μl) in triplicates. cAMP generation was started by adding 50 μl of 10 μM prostaglandin (PGE_2). 15 min later, the reaction was stopped by 1 N NaOH (50 μl) and placing on ice for 30 min. The sample was centrifuged (450*g*, 3 min), and levels of cAMP in the supernatant were determined by cAMP enzyme-linked immunosorbent assay kits (Amersham Pharmacia Biotech, UK).

Tumor Necrosis Factor-α Production from Human PBMC. Purified human PBMC (2×10^6/ml) suspended with RPMI 1640 medium, including 10% FBS, were incubated with each test compound at 37°C for 10 min before stimulation by lipopolysaccharide (LPS) (60 μg/ml). 20 h later, the reaction was stopped by adding 250 μl of 50 mM EGTA solution into the tubes and putting on ice. The tubes of sample were centrifuged (4°C, 250*g*, 10 min), and the amount of produced tumor necrosis factor-α (TNF-α) in the supernatant was measured by human TNF-α enzyme-linked immunosorbent assay kits (Amersham Pharmacia Biotech, UK).

Carrageenan-Induced Pleurisy in Rat. Male rats aged 6 to 7 weeks were used for carrageenan-induced pleurisy (CIP). The animals, which had been fasted overnight, were anesthetized with diethylether and given an injection of 1% (w/v) carrageenan solution in saline into the pleural cavity at a volume of 0.1 ml. 4 h after the injection, the animals were sacrificed by overanesthesia with chloroform, and pleural cavity lavage was performed with 2 ml of saline containing heparin (1 U/ml). The volume of lavage fluid recovered from the cavity was recorded, and the exuded volume in the cavity was determined. The total number of leukocytes was counted with an automatic cell counter (Celltac-α; Nihon-Koden, Japan). Each test compound was orally administered 30 min before the injection of carrageenan. The evaluation of each compound was carried out using an ED_{30} value, this being the dose achieving 30% inhibition (Grau et al., 1991).

CIP in Ferrets. We established a ferret CIP model by modifying the rat model. Ferrets, which had been fasted overnight, were given an injection of 1 ml of 1% carrageenan solution into the pleural cavity under ether anesthesia. 4 h later, the animals were sacrificed by overanesthesia with chloroform, and exuded cells in the pleural cavity were harvested with 5 ml of saline containing heparin (1 U/ml). Exudation volume in the cavity was determined from the weight of lavage fluid recovered, and leukocyte numbers were counted by Celltac-α. YM976, rolipram, and SB207499 were orally administered 30 min before the carrageenan injection.

Emetogenic Effects in Conscious Ferrets. The emetogenic activities of the compounds were examined in ferrets fasted overnight. Each test compound was orally administered at a volume of 3 ml/kg. The number of ferrets demonstrating emesis was recorded for 8 h from just after the administration. The emetogenic effects of the test compounds were expressed as the ratio of ferrets showing emesis/tested ferrets. The control group was treated with 0.5% MC.

Binding Assays and Enzyme Assays. YM976 was examined at 10 μM in these assays. Radioligand binding assays for the following receptors and channels were performed: adenosine A1 and A2; adrenergic α_1, α_2, and β; dopamine D1 and D2; GABA A; muscarinic M1, M2, and M3; nicotinic acetylcholine; serotonin; sigma; opiate; calcium channel type L and type N; chloride channel; potassium channel ATP-sensitive; Ca^{2+}-activated (voltage-dependent) and Ca^{2+}-activated (voltage-independent) and sodium site 1 and site 2. Inhibitory assays for the following enzymes were performed; cyclooxygenase-1 and -2, LTC_4 synthetase, 5-lipoxygenase, constitutive nitric-oxide synthase, inducible nitric-oxide synthase, pancreatic phospholipase A_2, elastase, protein kinase A, and protein kinase C.

Data Analysis. Data were expressed as the means with 95% confidence limits. Statistical significance of differences between means of groups was determined by Dunnett's multiple range test. Probabilities of $<.05$ were considered significant. Concentrations or doses causing 50 or 30% inhibition were determined by nonlinear curve fitting using SAS (SAS Institute Inc., Cary, NC). In case of cAMP elevation, EC_{200} was calculated as the concentration that doubled (200%) the level of cAMP induced by PGE_2.

Results

Inhibition of Purified PDE Isozymes. YM976 at 0.1 to 10 nM concentration dependently inhibited metabolic activity of PDE4 derived from human peripheral leukocytes, with an IC_{50} value of 2.2 (95% confidence limits: 1.7–2.9). Rolipram, RP73401, SB207499, and CDP840 also showed inhibitory effects on PDE4 in a concentration-dependent manner with IC_{50} values of 820 (540–1220), 0.43 (0.31–0.59), 117 (65–213), and 19 (16–23) nM, respectively (Table 1). The order of inhibitory activities of these compounds is RP73401 > YM976 > CDP840 > SB207499 $\gg$ rolipram.

YM976, rolipram, RP73401, SB207499, and CDP840 had no effects on the other PDE isozymes, PDE1, -2, -3, and -5, up to 100 (rolipram) or 1000 (the other compounds) times the IC_{50} values for PDE4 (Table 1).

The Mode of Inhibitory Action of YM976. The representative data from three separate experiments are shown in Fig. 2. Recombinant $PDE4_A$ exhibited standard Michaelis-Menten kinetics with a K_m of 2.1 μM and a V_{max} of 3.7 nmol/min/mg of protein. From the analysis of the Lineweaver-Burk plot, YM976 was shown to be a competitive inhibitor of PDE4. According to the Dixon plot, the K_i value of YM976 for $PDE4_A$ was 5.2 nM.

Enhancement of Intracellular cAMP Accumulation in Cell Line U937. YM976 concentration dependently enhanced the accumulation of cAMP in human U937 induced by PGE_2 with an EC_{200} value of 12 (5.1–24) nM. Based on EC_{200} values, YM976 was approximately eight times more potent than rolipram, which showed an EC_{200} value of 93 (14–306) nM (Fig. 3 and Table 2).

TNF-α Production from Human PBMC. PDE4 inhibitors are known to inhibit TNF-α production, and in this study all test compounds inhibited LPS-induced TNF-α production from human PBMC in a concentration-dependent manner. IC_{50} (95% confidence limit) values of YM976, rolipram, RP73401, SB207499, and CDP840 were 9.4 (5.4–16), 76 (40–131), 0.82 (0.50–1.3), 120 (91–150), and 19 (12–27) nM, respectively (Table 2). YM976 is stronger than rolipram and CDP840, but not as strong as RP73401. The order of TNF-α inhibition of these compound is the same as PDE4 inhibition, RP73401 > YM976 = CDP840 > rolipram = SB207499.

Carrageenan-Induced Pleurisy in Rats. The injection of carrageenan into the pleural cavity in control rats induced remarkable cell infiltration and obvious exudation. YM976, rolipram, and RP73401 inhibited the cell infiltration induced by carrageenan with ED_{30} values of 9.1, 10, 7.4 mg/kg, p.o., respectively (Fig. 3 and Table 3). However, CDP840 and

TABLE 1
IC_{50} values of YM976 and related compounds on PDE activity
Data were expressed as mean (95% confidence limits) of three to eight separate experiments. PDE4, PDE1, PDE3, and PDE5 were partially purified from human peripheral leukocytes, and PDE2 was isolated from rat ventricles.

Compound	IC_{50}				
	PDE4	PDE1	PDE2	PDE3	PDE5
	nM			*μM*	
YM976	2.2 (1.7–2.9)	>3	>3	>3	>3
Rolipram	820 (540–1220)	>100	>100	>100	>100
RP73401	0.43 (0.31–0.59)	>1	>1	>1	>1
SB207499	117 (65–213)	>300	>300	>300	>300
CDP840	19 (16–23)	>30	>30	>30	>30

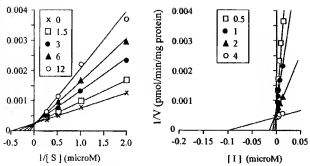

Fig. 2. Analysis of mode of inhibitory action by YM976 on cAMP hydrolysis by human recombinant PDE4$_A$. The data were representative of results from three separate experiments. Left, Lineweaver-Burk plot; the vertical axis shows the reciprocal of the reaction rate, and the horizontal axis shows the reciprocal of the substrate. Final concentrations of YM976 were 0, 1.5, 3, 6, and 12 nM. Right, Dixon plot; the vertical axis shows the reciprocal of the reaction rate, and the horizontal axis shows the concentrations of the inhibitor. Final concentrations of cAMP were 0.5, 1, 2, and 4 μM.

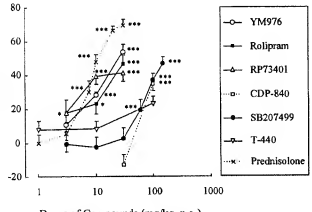

Fig. 3. Effects of YM976, the other PDE4 inhibitors, and prednisolone on the cell infiltration induced by carrageenan in rats. Test compounds were orally administered 30 min before the carrageenan injection. Data are expressed as the mean ± S.E. and represent the inhibition of cell infiltration in six animals. *P < .05 and ***P < .001 for difference between control group and each compound treatment group (Dunnett's multiple range test).

TABLE 2
Effects of YM976 and related compounds on cAMP elevation and TNF-α production

cAMP elevation was evaluated under the stimulation by PGE_2 (1 μM) in U937 cells. TNF-α production from human PBMC was induced by LPS (60 μg/ml). Each IC_{50} value was calculated from the percentage inhibition of control. Data are expressed as mean (95% confidence limits) of three to four separate experiments.

Compound	cAMP Elevation, EC_{200}	TNF-α Production, IC_{50}
	nM	
YM976	12 (5.1–24)	9.4 (5.4–16)
Rolipram	93 (14–306)	76 (40–131)
RP73401	1.1 (0.50–2.04)	0.82 (0.50–1.3)
SB207499	NT	120 (91–150)
CDP840	NT	19 (12–27)

NT, not tested.

SB207499 failed to exhibit the obvious inhibition at 30 mg/kg, p.o. Prednisolone dose dependently inhibited the cell infiltration with an ED_{30} value of 5.4 mg/kg, p.o.

Emetogenic Effects in Ferrets. No emesis was observed in vehicle-treated ferrets. YM976 did not cause emesis up to 10 mg/kg p.o. during 8 h after the administration, and at 30 mg/kg YM976 caused emesis in 33% of the ferrets. On the other hand, rolipram and RP73401 caused emesis at an oral dose of 3 mg/kg in 60 and 67% of the ferrets, respectively (Table 4). SB207499 and CDP840 also produced emesis in 67 and 100% of the ferrets at oral doses of 10 and 30 mg/kg, p.o., respectively.

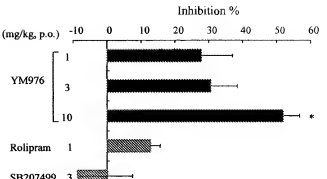

Fig. 4. Effects of YM976, rolipram, and SB207499 on cell infiltration of carrageenan-induced pleurisy in ferrets. Each compound was orally administered 30 min before the carrageenan injection. Data showed the inhibition percentage of each treatment when defined as 0% for the control group and as 100% for the saline group. Control and saline values were 28 ± 4 and 3 ± 0.4 millions/animal, respectively. *P < .05 for difference between control group and each compound treatment group (Student's t test or Dunnett's multiple range test, n = 3–6).

TABLE 3
Effective doses for 30% inhibition (ED_{30}) on cell infiltration and exudation in rat carrageenan-induced pleurisy

Effects on the pleurisy were evaluated 4 h after the carrageenan injection. Compounds were orally administered 30 min before carrageenan injection. Data are expressed as mean of six animals. The numbers in parentheses indicate 95% confidence limits.

Compound	ED_{30}	
	Cell Infiltration	Exudation
	mg/kg, p.o.	
YM976	9.1 (7.2–12)	18 (12–35)
Rolipram	10 (4.8–24)	7.1 (3.4–12)
RP73401	7.4 (2.8–14)	>30
SB207499	87 (61–140)	120 (87–200)
CDP840	>30, <100	>100
Prednisolone	5.4 (4.5–6.3)	7.9 (6.0–11)

Carrageenan-Induced Pleurisy in Ferrets. The anti-inflammatory effects of YM976, rolipram, and SB207499 were evaluated in ferrets. YM976 dose dependently inhibited the infiltration of leukocytes at doses of 1–10 mg/kg, p.o. (Fig. 4). In addition, YM976 also produced a significant inhibition of the exudation into the cavity at 3 and 10 mg/kg. On the other hand, rolipram and SB207499 failed to reduce the cell infiltration at 3 mg/kg, which was the maximal dose to cause no emesis in ferrets. The effects of these compounds on exudation were the same as those on cell infiltration (data not shown).

Discussion

There have been many PDE4 inhibitors reported so far. Although some of them were reported to be less emetic, no compounds have been evidenced to show dissociation of emesis from anti-inflammatory activity in vivo. This is the first

TABLE 4
Emetogenic effects of YM976 and related compounds in ferrets
The emetogenic activities of test compounds were assessed in fasted ferrets. Each test compound was orally administered at a volume of 3 ml/kg. From just after the administration, the number of ferrets demonstrating emesis was recorded for 8 h. The ratio (%) was determined by division of the number of ferrets showing emesis by that of ferrets tested.

Compound	Dose	No. of Animals (Emesis/Tested)	Ratio
	mg/kg		%
0.5% MC		0/8	0
YM976	10	0/8	0
	30	2/6	33
	100	2/4	50
Rolipram	1	0/5	0
	3	3/5	60
	10	4/5	80
RP73401	1	0/3	0
	3	4/6	67
	10	3/3	100
SB207499	3	0/3	0
	10	2/3	67
	30	2/3	67
	100	3/3	100
CDP840	10	0/1	0
	30	5/5	100

report showing apparent dissociation of the anti-inflammatory activity from emetic effects in experimental levels.

YM976 is a novel type of PDE4 inhibitor synthesized based on a lead compound found by random screening. The structure is totally different from the existing PDE4 inhibitors, in terms of lacking the 3-cyclopentyloxy-4-methoxyphenyl group, which is shared by rolipram (Underwood et al., 1993), RP73401 (Naline et al., 1996), SB207499 (Underwood et al., 1998), and CDP840 (Holbrook et al., 1996). In vitro cell-free experiments showed that YM976 was a strong and competitive inhibitor of PDE4. Its inhibitory effect on PDE4 activity was approximately 500-fold stronger than that of rolipram, and four times weaker than that of RP73401, which is one of the strongest PDE4 inhibitors (see Table 1). YM976 showed no activities against the other PDE isozymes such as PDE1, -2, -3, and -5. Additionally, YM976 did not exhibit significant activities for the other receptors, channels, or enzymes at 10 μM (data not shown). These results indicate that YM976 is a specific inhibitor for PDE4.

YM976 potentiated the intracellular cAMP level in U937 cells. The ratio of an EC_{200} value for intracellular cAMP versus an IC_{50} for PDE4 is 12:2, suggesting that YM976 is well permeable to the cell membranes. Next, we examined the effect of YM976 on the cell function relevant to cAMP. The production of TNF-α from human PBMC is known to be related to intracellular cAMP levels and strongly inhibited by PDE4 inhibitors (Seldon et al., 1998). YM976 concentration dependently decreased TNF-α production with an IC_{50} value of 11 nM. This inhibitory concentration is almost the same as the EC_{200} value for the cAMP elevation. Thus, the inhibitory effect of YM976 on TNF-α production is probably produced via increased cAMP by inhibiting PDE4.

Rolipram also potentiated the elevation of cAMP induced by PGE_2 with an EC_{200} value of 93 nM, and inhibited TNF-α production with an IC_{50} value of 83 nM. These effective concentrations, however, are lower than the IC_{50} value for PDE4 enzyme (IC_{50} = 820 nM). Although the precise mechanism has not been elucidated, rolipram may have other effects, such as the activation of adenylate cyclase and/or G_s protein, which could stimulate cAMP production.

Next, we evaluated the in vivo effects of YM976. PDE4 inhibitors are known to produce multiple anti-inflammatory effects and have therapeutic potential for asthma (Schudt et al., 1995), rheumatoid arthritis (Nyman et al., 1997; Ross et al., 1997), and nephritis (Tanahashi et al., 1999). Because drugs for chronic inflammatory diseases should be administered daily, orally active compounds are desirable as therapeutics. Thus, we estimated the anti-inflammatory effects of YM976 by the oral route. Carrageenan-induced pleurisy is an acute inflammatory model, which is characterized by leukocyte and fluid accumulation associated with extravasation of plasma protein. PDE4 are distributed in endothelial cells as well as neutrophils (Derian et al., 1995; Pryzwansky et al., 1998), which are the predominant type of infiltrated cells in this model (Vinegar et al., 1982). A PDE4 inhibitor is considered to inhibit leukocyte infiltration and fluid exudation into the pleural cavity by several modes of action such as reduction of the extravasation caused by direct inhibition of endothelial contraction (Ortiz et al., 1996; Adamson et al., 1998), inhibition of the production of chemotactic factors like leukotriene B_4 (Denis and Riendeau, 1999), inhibition of neutrophil chemotactic activity, and inhibition of adhesion molecule expression on endothelial cells (Armstrong, 1995; Morandini et al., 1996; Berends et al., 1997).

Carrageenan is known to activate the complement system and to induce histamine and serotonin release causing leukocyte migration and extravasation (Capasso et al., 1975). Because YM976 has no direct effect on the complement system and chemical mediators, the inhibition by YM976 may be exhibited through PDE4 inhibition. YM976 is as effective as RP73401 in this model (Table 3), indicating that YM976 is well absorbed from the gastrointestinal tract. PDE4 inhibitors are generally known to cause emesis as a major adverse effect. In fact, some PDE4 inhibitors are reported to induce emesis when systemically administered to humans (Bertolino et al., 1988; Hebenstreit et al., 1989; Schmidt et al., 1999), which limits their therapeutic potential (Heaslip and Evans, 1995). To estimate the safety windows, the emetogenicity and anti-inflammatory effects should be evaluated in the same animal species.

Thus, we examined the effects of YM976 and other PDE4 inhibitors using the ferret, which is one of the most suitable animal species for experiments in gastrointestinal actions. Rolipram and SB207499 did not show suppression on cell infiltration at the dose demonstrating no emesis in ferrets, although SB207499 was reported to produce no emesis at doses that showed anti-asthmatic effects in clinical trials (Torphy et al., 1999). On the contrary, YM976 inhibited carrageenan-induced cell infiltration at doses of 1 to 10 mg/kg, p.o., whereas it produced no emesis up to 10 mg/kg, p.o. These results suggest that YM976 has a weaker emetogenicity and a broader safety window than other PDE4 inhibitors such as SB207499. A compound showing apparent dissociation of anti-inflammatory effect from emesis in the same species, ferrets, has not been reported. It is important that higher doses could be administered to patients due to a wide safety window. The clinical trial of YM976 may propose the association of PDE4 with the pathological process in several inflammatory diseases.

It is important to elucidate the reason why YM976 has a less emetic effect. Although the precise mechanism remains to be solved, there are three possible mechanisms for reduced

emetogenicity, i.e., the affinity to high-affinity rolipram binding site (Sr), selectivity to PDE4 subtypes, and poor brain penetration. First, it was reported that PDE4 enzyme has Sr as well as a catalytic site and that the affinity to Sr was related to the emetogenicity (Duplantier et al., 1996). Second, PDE4 has at least four subtypes (A, B, C, and D) and the selectivity to these subtypes may be associated with the emetogenicity. However, the relationship between the subtypes and the functions/activities is not clear. Finally, if the emetogenicity by PDE4 inhibitors is related to the central nervous systems, the concentration of the compound in the brain is important. The emetogenicity may contribute to brain penetration of the compound. Because YM976 has a novel chemical structure, it should have different characteristics from the other compounds, such as rolipram, RP73401, SB207499, and CDP840.

In conclusion, these studies demonstrate that YM976 is a potent and selective PDE4 inhibitor having a novel structure, and, more importantly, the first compound indicating dissociation of anti-inflammatory activities from emetic effects in the same animal species in comparison with rolipram and SB207499. The dissociation observed by YM976 may offer an effective approach for a new generation of PDE4 inhibitors.

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